JOURNAL OF BACTERIOLOGY, Nov. 1967, p. 1802-1803

Vol. 94, No. 5
Printed in U.S.A.

Induction of Mutations by Nitrous Acid on Denatured *Haemophilus influenzae* Deoxyribonucleic Acid Assayed Directly by Single-stranded Transformation

EDITH H. POSTEL AND SOL H. GOODGAL

Department of Microbiology, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Received for publication 8 August 1967

Nitrous acid has been shown to be an effective mutagenic agent on transforming deoxyribonucleic acid (DNA) of *Haemophilus influenzae* (E. E. Horn and R. M. Herriott, Proc. Natl. Acad. Sci. U.S. **48**:1409, 1962; S. H. Goodgal and E. H. Postel, Science **148**:1095, 1965). Unlike the transforming DNA of *Diplococcus pneumoniae* (R. Litman and H. Ephrussi-Taylor, Compt. Rend. **249**:838, 1959) and *Bacillus subtilis* (C. Anagnostopoulos and I. P. Crawford, Proc. Natl. Acad. Sci. U.S. **47**:378, 1961), however, the DNA of *H. influenzae* had to be denatured in order for nitrous acid to produce mutant markers. After denaturation and nitrous acid treatment, it was necessary to renature the DNA to show the transforming ability of the mutant markers (E. E. Horn and R. M. Herriott, Proc. Natl. Acad. Sci. U.S. **48**:1409, 1962). The recent development of a system for transformation with single-stranded DNA in *H. influenzae* (E. H. Postel and S. H. Goodgal, J. Mol. Biol. **16**:317, 1966; E. H. Postel and S. H. Goodgal, J. Mol. Biol. **27**:247, 1967; S. H. Goodgal and E. H. Postel, J. Mol. Biol. **27**:261, 1967) has made it possible to eliminate the renaturation step and to assay directly for the induction of mutations on single-stranded DNA. An experiment of this nature is described below and the results confirm the conclusion made by Horn and Herriott (Proc. Natl. Acad. Sci. U.S. **48**:1409, 1962) that nitrous acid produces mutations by acting directly on the bases of single-stranded DNA of *H. influenzae*.

The experimental procedure involved the use of DNA carrying a novobiocin resistance marker (C_{25}) which made it possible to follow the inactivation of the treated DNA, as well as the appearance of induced mutations. The DNA was heat-denatured, quickly cooled, treated with nitrous acid, and assayed for the loss of the C_{25} marker and for the appearance of streptomycin and kanamycin markers in the *denatured transformation assay*. In addition, portions of the treated DNA were renatured and assayed in the *native transformation assay*. (See figure legend for details of the experiment). Figure 1A shows the loss of the C_{25} marker activity as a function of HNO_2 treatment as assayed before and after renaturation. These assays were done at saturating concentrations of DNA. The same results were obtained with concentrations in the linear response region. The inactivation of the C_{25} marker assayed after renaturation was slower than treated DNA assayed directly in the single-stranded system. One possible interpretation of these results is that the inactivated marker which had been renatured undergoes repair inside the cells, whereas single-stranded DNA is not repaired. The dark repair of ultraviolet damage of cells and transforming activity has been described for *H. influenzae* (R. F. Day, Ph.D. Thesis, Pennsylvania State University, University Park, 1967).

The induction of mutations to streptomycin and kanamycin resistance is shown in Fig. 1B. These results demonstrate that mutations on denatured DNA may be scored directly without renaturation in the single-stranded assay system, as well as after renaturation in the native assay system. The kinetics of mutation induction appeared similar for both assay systems, although the absolute numbers of mutants to both kanamycin and streptomycin resistance was greater for the renatured DNA (Fig. 1B). This difference may be attributed on the one hand to the higher transforming efficiency of native DNA, normally two- to fivefold better than denatured DNA (E. H. Postel and S. H. Goodgal, J. Mol. Biol. **27**:247, 1967), and on the other hand to the higher specific transforming activity of the renatured DNA by virtue of its less extensive inactivation (Fig. 1A).

The fact that nitrous acid produced mutations only on denatured DNA in *H. influenzae* suggested that its action was a direct attack on the exposed bases of the denatured DNA (E. E. Horn and

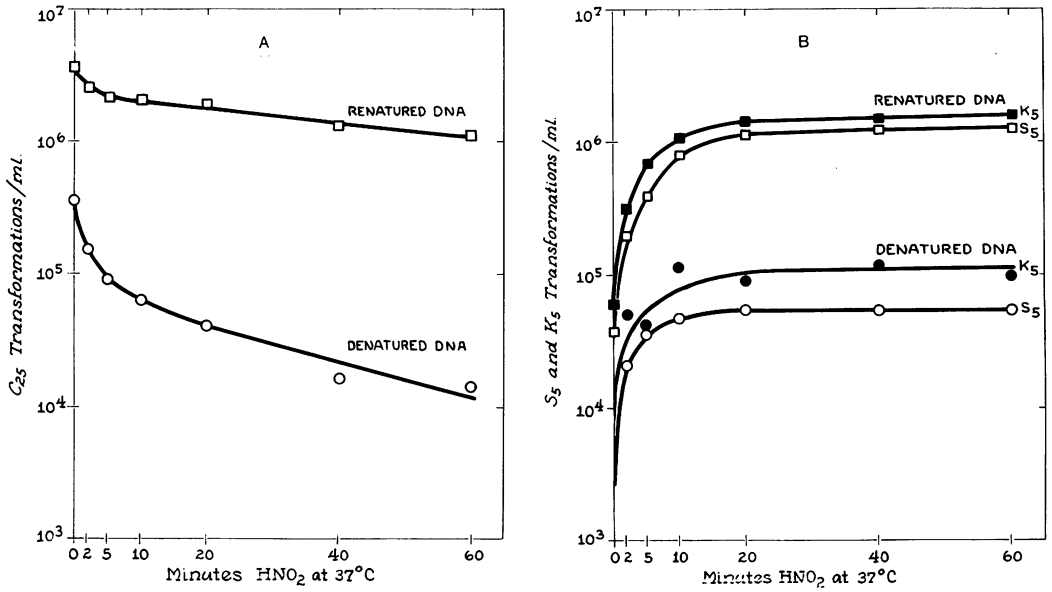

FIG. 1. *Effect of nitrous acid treatment. DNA (1.2 ml, 200 μg/ml of C_{25}) was heat-denatured for 10 min and quickly cooled. To 0.9 ml of this DNA, 0.9 ml of nitrite buffer (2 M $NaNO_2$ in 0.08 M acetate buffer at pH 4.2) was added. The mixture was incubated at 37 C and at intervals 0.2-ml samples were added to 1.8 ml of 0.02 M Na_2HPO_4 in 0.15 M NaCl to neutralize the nitrous acid. (The final concentration of DNA was 10 μg/ml). Assay for single-stranded transforming activity was as follows (see E. H. Postel and S. H. Goodgal, J. Mol. Biol.* **27**:*247, 1967, procedure C). To 3 ml (8×10^8 cells/ml) of peptone (Difco) buffer (pH 4.4) plus 3×10^{-4} M ethylenediaminetetraacetic acid, 0.1 ml of the above neutralized DNA sample was added. The final DNA concentration was 0.33 μg/ml. After 5 min of incubation at 37 C, 0.03 ml of 0.1 N NaOH was added to bring the pH of the mixture to approximately 6.6. After 20 min of incubation at 34 C, the culture was plated for transformants. The plates were incubated for 90 min at 37 C and then overlayed with the appropriate concentrations of antibiotics (25 μg of novobiocin per ml for the intrinsic marker and 5 μg of streptomycin and 5 μg of kanamycin per ml for the mutant markers). Procedures for screening of the mutants, with appropriate controls, were carried out as described previously (E. E. Horn and R. M. Herriott, Proc. Natl. Acad. Sci. U.S.* **48**:*1409, 1962; S. H. Goodgal and E. H. Postel, Science* **148**:*1095, 1965). To assay for double-stranded (renatured) transforming activity, 1 ml of each neutralized sample was renatured by holding it in a water bath (67 C) for 2 hr. To 3 ml (8×10^8 cells/ml) of brain heart infusion broth, 0.1 ml of the renatured DNA was added (final DNA concentration, 0.33 μg/ml); after 20 min of incubation at 34 C, the cultures were treated the same way as described above for the assay of single-stranded transforming activity.*

R. M. Herriott, Proc. Natl. Acad. Sci. U.S. **48**:1409, 1962). Therefore, integration of nitrous acid-treated DNA would be expected to incorporate altered bases directly, since transformation with native DNA (N. Notani and S. H. Goodgal, J. Gen. Physiol. **49**:197, 1966), as well as with denatured DNA (S. H. Goodgal and E. H. Postel, J. Mol. Biol. **27**:261, 1967), involves the integration of only one strand of the donor DNA. Mechanisms which induce breakdown and resynthesis of altered bases into DNA may be operative during the integration process and cannot be rigorously excluded from participation in the process of mutagenesis with nitrous acid. However, the simplest interpretation suggests that a direct incorporation of DNA with altered bases is responsible for mutations. This conclusion is supported by evidence which demonstrates that the expression and replication of nitrous acid-induced transformants does not differ from that of normal transformants (S. H. Goodgal and E. H. Postel, Science **148**:1095, 1965).

We acknowledge the excellent technical assistance of Nancy Stanton.

This investigation was supported by Public Health Service grant AI-04557 from the National Institute of Allergy and Infectious Diseases.